# Transcutaneous Immunization with Bacterial ADP-Ribosylating Exotoxins, Subunits, and Unrelated Adjuvants

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We have recently described a needle-free method of vaccination, transcutaneous immunization, consisting of the topical application of vaccine antigens to intact skin. While most proteins themselves are poor immunogens on the skin, we have shown that the addition of cholera toxin (CT), a mucosal adjuvant, results in cellular and humoral immune responses to the adjuvant and coadministered antigens. The present study examines the breadth of adjuvants that have activity on the skin, using diphtheria toxoid (DTx) and tetanus toxoid as model antigens. Heat-labile enterotoxin (LT) displayed adjuvant properties similar to those of CT when used on the skin and induced protective immune responses against tetanus toxin challenge when applied topically at doses as low as 1 μg. Interestingly, enterotoxin derivatives LTR192G, LTK63, and LTR72 and the recombinant CT B subunit also exhibited adjuvant properties on the skin. Consistent with the latter finding, non-ADP-ribosylating exotoxins, including an oligonucleotide DNA sequence, as well as several cytokines (interleukin-1β [IL-1β] fragment, IL-2, IL-12, and tumor necrosis factor alpha) and lipopolysaccharide also elicited detectable anti-DTx immunoglobulin G titers in the immunized mice. These results indicate that enhancement of the immune response to topical immunization is not restricted to CT or the ADP-ribosylating exotoxins as adjuvants. This study also reinforces earlier findings that addition of an adjuvant is important for the induction of robust immune responses to vaccine antigens delivered by topical application.

Worldwide rates of immunization for diphtheria, pertussis, tetanus, polio, measles, and tuberculosis have increased dramatically over the last 20 years. Barriers to mass immunization vary widely among economic markets, but common constraints include the requirement for trained personnel, the association of vaccines with needle-related diseases and injuries, and cold-chain and transport issues. Development of less-invasive and more readily administered vaccines has thus become a priority for public health agencies and is associated with an emergence of new technologies in the areas of vaccine delivery vehicles and routes of administration (1, 15, 19, 25).

Researchers have recently described transcutaneous immunization (TCI), a needle-free method for delivering vaccines to the host by simple application of adjuvant and antigen to the exterior skin surface (15, 16, 17, 18, 19, 29). Our previous studies demonstrated that cholera toxin (CT) applied to the skin could be used as an adjuvant to elicit serum immunoglobulin G (IgG) responses against itself and coadministered antigens, including bovine serum albumin, diphtheria toxoid (DTx), hen egg lysozyme, tetanus fragment C, and tetanus toxoid (TTx). The humoral responses elicited by TCI were boosted by sequential applications, and antibodies are detectable in lung and stool exudates (16, 17), suggesting the potential for eliciting mucosally relevant immunity by this method. Moreover, we have reported that application of CT and DTx onto the skin induces a proliferative response against DTx in the spleen and draining lymph node tissue that is associated with antigen-specific CD4^{+} T-cell activation (18, 29). Similar observations have been made with hen egg lysozyme, a soluble *Leishmania* parasite extract, and recombinant malarial pro-

teins (T. Scharton-Kersten, unpublished observations). Based on these results, human trials have been initiated to evaluate the feasibility of this technology for safely and effectively administering human use vaccine antigens.

Our initial studies of TCI focused on the adjuvant activity of CT, an 86-kDa member of the bacterial ADP-ribosylating exotoxin family that is composed of a single proteolytically activated A chain (27 kDa) and five B chains (12 kDa). The mucosal adjuvant function of CT is well described for numerous experimental and vaccine antigens administered by the oral, intranasal, and intrarectal routes (2, 12, 26), although the diarrhea associated with the A subunit activity has prevented its use in human vaccines (24). One concern with the TCI method is the use of high doses of the "toxic" CT molecule as an adjuvant. However, topical application of CT does not appear to result in the side effects that may occur with its oral, intranasal, and parenteral uses (6, 15, 16).

The primary goal of the present study was to identify the breadth of compounds that might be employed as adjuvants for TCI. Heat-labile enterotoxin (LT) was an obvious candidate adjuvant for TCI since it shares 82% amino acid homology with CT and has been shown to induce quantitatively similar mucosal immune responses compared to CT following oral and intranasal administration (32). In contrast to CT, LT may be less prone to the induction of diarrhea in humans (24, 27), and it has been suggested that LT induces a stronger Th1 response than CT (32). Comparison of CT and LT applied to the skin with DTx revealed that both proteins induced qualitatively and quantitatively similar responses against DTx in the serum. Anti-DTx IgG1 was the predominant subclass induced in both groups, with a weaker but clearly detectable IgG2a response. A second series of experiments using TTx as the coadministered antigen revealed that a dose of LT as low as 1 μg could be combined with as little as 5 μg of TTx to induce a protective immune response.

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Our results with CT and LT indicate that the ADP-ribosylating exotoxins are potent topical adjuvants. However, while these studies were consistent with an association between the ADP-ribosylating activity and adjuvant activity on the skin, it was not clear whether the enzyme function was absolutely required during TCI. To address this question, a wide variety of structurally dissimilar compounds were applied to the skin, and the induction of antigen-specific immune responses was assayed. Two broad classes of compounds were evaluated: derivatives of CT and LT with attenuated ADP-ribosylating activity (CT B subunit [CTB], LTR192G [4], LTK63 [10], and LTR72 [13]) and molecules that lacked the CT A subunit or its enzyme function entirely (cytokines, DNA, alum, and bacterial products). Most of the compounds evaluated were capable of eliciting a serum antibody response against the coadministered antigen, indicating that ADP-ribosylating activity is not essential for topical adjuvant function.

## MATERIALS AND METHODS

**Animals.** BALB/c and C57BL/6 mice were obtained from Jackson Laboratories (Bar Harbor, Maine). Mice (6 to 10 weeks old) were maintained in pathogen-free conditions and fed rodent chow and water ad libitum.

**Antigens and adjuvants.** CT and DTx, purified CTB (pCTB), recombinant CTB, *Salmonella enterica* serovar Minnesota R595 lipid A, and tetanus toxoid (TT) were purchased from List Biologicals (Campbell, Calif.). LT and TTx were provided by Berne Laboratories (Coral Gables, Fla.). Complete Freund adjuvant (CFA), interleukin-1β (IL-1β) fragment, IL-2, IL-12, *Escherichia coli* O127:B8 LPS, muramyl dipeptide (MDP ["adjuvant peptide"]), *Salmonella enterica* serovar Typhimurium lipopolysaccharide (LPS), *Shigella flexneri* 1A LPS, and *Vibrio cholerae* serotype Inaba LPS were purchased from Sigma Chemicals (St. Louis, Mo.). Tumor necrosis factor alpha (TNF-α) was procured from Endogen Corp. (Woburn, Mass.). Alum was acquired from Rehcis, Inc. (Berkeley Springs, N.J.). Oligonucleotide 1826 (also referred to as CpG) and control oligonucleotide were obtained from Oligos, Etc. (Wilsonville, Oreg.). LTK63 and LTR72 were generous gifts of Rino Rappuoli (Chiron S.p.A, Siena, Italy), and LTR192G was a gift of John Clements (Tulane University Medical Center, New Orleans, La.).

**Immunizations.** Mice were shaved on the dorsum with a no. 40 clipper and rested for 48 h. Groups of 3 to 15 mice were anesthetized in the hind thigh intramuscularly with 110 mg of ketamine per kg of body weight mixed with 11 mg of xylazine per kg during the immunization procedure to prevent grooming. The shaved skin was hydrated by wetting with water-drenched gauze for 5 min and lightly blotted with dry gauze prior to immunization. Then 50 to 100 μl of immunizing solution was placed on the shaved skin over an approximately 2-cm² area for 2 h. The mice were then extensively washed, tail down, under running, lukewarm tap water for approximately 30 s, patted dry, and washed again.

**TTx challenge.** Mice were challenged with TTx as previously described [11]. The mice were injected subcutaneously in the abdomen with 387 μg of TTx in nutrient broth-borate buffer (1:2) and then observed for survival for 12 days.

**Antibody assays.** Antibody levels against CT, DTx, LT, and TTx were determined using enzyme-linked immunosorbent assay (ELISA). For total IgG determinations, Immulon-2 polystyrene plates (Dynatech Laboratories, Chantilly, Va.) were coated with 0.1 μg of antigen per well in saline, incubated at room temperature overnight, blocked with 0.5% casein with 1% Tween 20, and washed; serial dilutions of serum were then applied, and the plates were incubated for 2 h at room temperature. Specific IgG (heavy plus light chains [H+L]) antibody was detected using horseradish peroxidase-linked goat anti-mouse IgG (H+L; Bio-Rad, Richmond, Calif.) and revealed after 30 min using 2,2′-azinobis(3-ethylbenzthiazoline sulfonic acid) substrate (ABTS; Kirkegaard and Perry, Gaithersburg, Md.), and the reaction was stopped using 1% sodium dodecyl sulfate. IgG subclass antibodies were measured as previously described [14]. The plates were read at 405 nm. Results of the total IgG assays are reported in ELISA units (EU), which are defined as the inverse dilution of the sera that yields an optical density (OD) of 1.0 at 405 nm. Prebleed and control values are reported in EU (OD × dilution) but were determined as duplicate samples at a single (1/100) dilution.

Lung washes were obtained from immunized mice as previously described [30]. The mice were sacrificed and exsanguinated by cardiac puncture, the tracheas were transected, 22-gauge polypropylene tubing was inserted, and phosphate-buffered saline was infused to gently inflate the lungs. The infused material was then withdrawn and reinfused for a total of three cycles and stored at −20°C. In previous studies, dipstick hemoglobin testing in samples from more than 100 mice indicated the absence of contaminating blood in the lung wash (G. R. Matyas, unpublished observations).

**Statistical analysis.** Unless otherwise indicated, the data shown are the geometric means of values from individual animals. Comparisons between antibody titers in groups were performed by using an unpaired, two-tailed Student's *t* test

using Microsoft Excel (Microsoft Corp., Redmond, Wash.), and *P* values of <0.05 were regarded as significant.

## RESULTS

**Adjuvant properties of CT and LT following topical application to the skin with DTx.** It has been previously shown that CT is an effective adjuvant on the skin, inducing serum and mucosal immune responses against itself and coadministered antigens such as DTx [15, 16, 17, 18, 29]. LT is also a member of the bARE family that is produced by a distinct bacterial strain, *E. coli* rather than *V. cholerae*, but is similar to CT in its amino acid sequence and tertiary structure. To determine whether LT might function as an adjuvant on the skin, C57BL/6 mice were immunized topically three times at 4-week intervals with DTx in the presence of 100 μg of CT or LT, and the development of anti-DTx IgG titers was measured in the serum. Comparable anti-LT or -CT titers were observed in groups receiving adjuvant alone or antigen and adjuvant (Fig. 1A and B), indicating that the addition of the antigen does not interfere with the immunogen function of these proteins. Both CT- and LT-adjuvanted groups displayed readily detectable anti-DTx IgG titers after the second immunization (Fig. 1C and D). After the third immunization at 8 weeks, a 10- to 100-fold boost was observed, resulting in geometric mean titers in serum of approximately 10,000 anti-DTx IgG EU in both groups. The EU value is a conservative estimate of titers defined as the inverse of the dilution resulting in an $OD_{405}$ of 1.0. Sera of control animals immunized with DTx or adjuvant (CT or LT) alone contained <25 EU of DTx-specific IgG at all time points.

Although the magnitude of the serum anti-DTx IgG titers was comparable in animals treated with LT or CT, it was possible that the quality of immunity induced by these adjuvants might differ. To test this concept, serum from mice immunized with CT and DTx or LT and DTx was analyzed for antigen-specific IgG1, IgG2a, and IgG2b titers (Table 1). Of the IgG subclasses, the IgG1 titers were the highest in both groups. A consistent but relatively lower IgG2a response also developed in both groups. IgG2b levels were negligible. Thus, similar IgG subclass profiles were observed in mice immunized with DTx and either CT or LT.

**Immunization of mice with LT and TTx induces a protective anti-TTx response.** Our standard adjuvant-antigen dose for immunizing mice and screening antigens and adjuvants has been 100 μg of each protein. These doses were chosen empirically without a formal analysis of the lower limits required for the topical vaccination. To address the possibility of utilizing lower antigen and adjuvant doses for immunization, a range of doses of TTx (0, 1, 5, 10, 25, and 50 μg) and LT (0, 1, 5, 10, 25, 50, and 100 μg) were applied to mice, and the resulting antigen-specific titers were assessed in the serum 2 weeks after the third immunization. None of the mice immunized with adjuvant alone displayed detectable anti-TTx titers. Similarly, mice immunized with TTx alone, in the absence of LT, exhibited very low or undetectable anti-TTx titers regardless of the antigen dose, although the higher doses (25 and 50 μg) clearly elicited some TTx responders (Fig. 2). However, the addition of even 1 μg of adjuvant induced an anti-TTx antibody response that was several orders of magnitude higher than that observed in the group exposed to the antigen alone (Fig. 2). Both the antigen and the adjuvant doses clearly influenced the magnitude of the serum anti-TTx responses, with a threshold for consistent responses achieved at TTx doses of ≥5 μg and at LT doses of ≥1 μg.

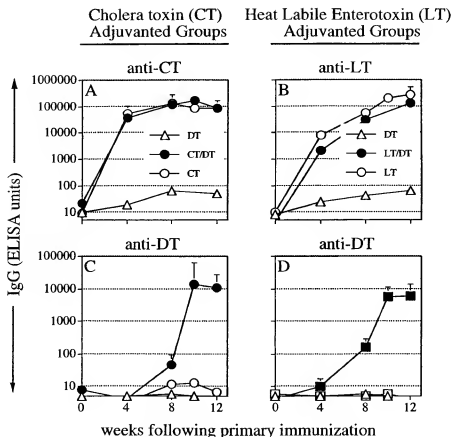The biological significance of the anti-TTx responses elicited

FIG. 1. Antibody response using CT or LT as adjuvants with DTx. C57BL/6 mice ($n = 5$) were immunized on the skin with CT and DTx (100 μg each), LT and DTx (100 μg each), or CT, LT, or DTx alone (100 μg each) at 0, 4, and 8 weeks. Sera collected 4 weeks after each immunization were assayed for DTx-specific IgG by ELISA as described in Materials and Methods. Symbols in panel D: ■, DT/LT; □, LT alone; △, DT alone. The geometric mean and the standard error of the mean (SEM) are shown for each group.

was evaluated by challenging the mice with a lethal dose of tetanus toxin (~387 pg/mouse) and monitoring survival (Table 2). All ($n = 42$) of the mice which failed to receive TTx during the immunization succumbed within 5 days of the challenge. In contrast, mice receiving ≥5 μg of TTx and 1 to 100 μg of LT were consistently protected, displaying 80 to 100% survival at 12 days following the challenge. As predicted by the serum anti-TTx titers, survival was sporadic in the groups immunized with 25 or 50 μg of TTx without adjuvant and in the groups receiving 1 μg of TTx with adjuvant.

TABLE 1. Serum anti-DTx subclass analysis in mice immunized with CT and DTx or with LT and DTx[a]

| Immunization group | Mean IgG concn (μg/ml) | | |
|---|---|---|---|
| | IgG1 | IgG2a | IgG2b |
| CT+DTx | 36.5 (1.9–584.1) | 13.7 (0.9–159.1) | 0.5 (0.3–0.7) |
| LT+DTx | 44.3 (4.7–394.8) | 8.9 (1.2–23.0) | 0.5 (0.1–2.1) |

[a] C57BL/6 mice ($n = 5$) were immunized on the skin with CT and DTx (100 μg each) or LT and DTx (100 μg each) at 0, 4, and 8 weeks. Sera collected 4 weeks after the final immunization were assayed for DTx-specific IgG1, IgG2a, and IgG2b by ELISA as described in Materials and Methods. Sera from unimmunized mice or animals immunized with adjuvant or antigen alone contained undetectable levels of DTx-specific antibody (≤1, 0.5, and 0.05 ng/ml for IgG, IgG2a, and IgG2b, respectively). Values in parentheses are ranges.

**Use of genetically modified LT, LTR192G, LTK63, and LTR72, as topical adjuvants.** LT is composed of two subunits: an A subunit containing the ADP-ribosylation activity and the B subunit that contains the cell surface binding region (7). The ADP-ribosylation activity has been associated with its oral and nasal adjuvant function and the toxicity of the holotoxin. The possible dissociation of adjuvant activity from gastrointestinal toxicity is an active area of research that has resulted in the generation of several LT mutants that maintain significant adjuvant activity with reduced side effects. We have screened three of these mutants for their topical adjuvant potential using a 100-μg dose of adjuvant. In the first experiment (Fig. 3), mice were immunized with DTx and native LT or LTR192G, a toxin derivative with a mutation in the trypsin-sensitive loop that joins the A1 and A2 moieties of the A subunit (9). Serum anti-DTx IgG titers determined after the third immunization were indistinguishable between the groups receiving holotoxin or LTR192G. In a second experiment (Fig. 3), LTK63 and LTR72, two active-site mutants with reduced ADP-ribosylating activity and toxicity (10, 13) were screened as adjuvants using DTx as the coadministered antigen. After a series of three immunizations, the serum anti-DTx IgG titers were elevated in both groups compared to the prebleed titers. All three LT derivatives exhibited topical adjuvant functions, although direct comparison of the three mutant adjuvants was not done and experiment 2 used only historical controls.
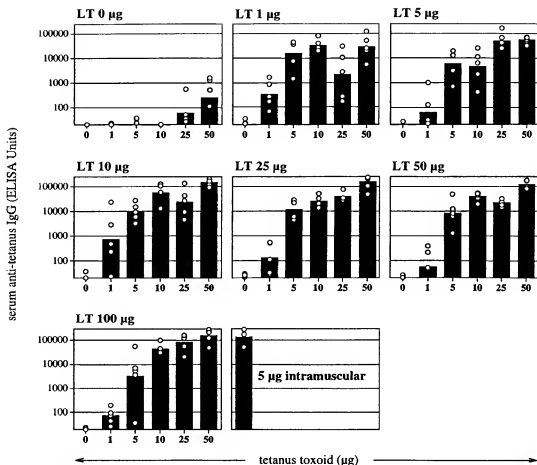
FIG. 2. Anti-TTx response in mice immunized with various doses of antigen and adjuvant. C57BL/6 mice (*n* = 5) were immunized on the skin with LT (doses indicated above each panel) and TTx (doses indicated on the *x* axis) at 0, 4, and 7 weeks. The intramuscular group was immunized with alum and TTx (5 µg) doses at 0, 4, and 8 weeks. Sera collected 2 weeks after the final immunization were assayed for TTx-specific IgG by ELISA as described in Materials and Methods. The geometric mean (bar) and individual values (open circles) are shown for each group.

**Adjuvant activity of the recombinant CTB subunit on the skin.** Oral and intranasal studies using the CTB subunit and coadministered antigens have yielded conflicting results regarding CTB adjuvant activity (3). The source of the CTB used by different laboratories, purified or recombinant, is one factor that has impeded interpretation of research addressing this issue. To address this question in our model, the adjuvant effects of topically applied CTB were evaluated using purified

CTB (pCTB), recombinant CTB (rCTB), or rCTB spiked with 1% holotoxin coadministered with DTx. Animals were immunized at 0, 4, and 8 weeks, and the development of elevated DTx-specific IgG was monitored in the serum for 12 weeks. Relative to the cohort receiving DTx alone, all of the adjuvants

TABLE 2. Percent survival after challenge with TT of mice immunized on the skin with LT and TTx[a]

| LT dose (µg) | % Survival with TTx dose of: | | | | | |
|---|---|---|---|---|---|---|
| | 0 µg | 1 µg | 5 µg | 10 µg | 25 µg | 50 µg |
| 0 | 0* | 20 | 0 | 0 | 20 | 60 |
| 1 | 0† | 40 | 100 | 80 | 80 | 100 |
| 5 | 0 | 20 | 100 | 80 | 100 | 100 |
| 10 | 0 | 40 | 100 | 100 | 100 | 100 |
| 25 | 0 | 0 | 100† | 100 | 100† | 100 |
| 50 | 0† | 0 | 100 | 100 | 100 | 100 |
| 100 | 0 | 0 | 80 | 80 | 100 | 100 |

[a] All groups contain 5 mice except as indicated (∗, *n* = 14; †, *n* = 4). Mice (*n* = 5 to 14) were challenged with 387 pg of TT by the subcutaneous route 4 weeks after the third immunization. Animals which were not protected succumbed within 3 to 7 days of toxin exposure.
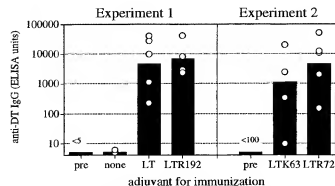


FIG. 3. Anti-DTx antibody response using LT or LT mutant toxins as adjuvants. C57BL/6 mice (*n* = 5) were immunized on the skin with LT or LT mutants and DTx (100 µg each) at 0, 4, and 8 weeks. Sera collected 4 weeks after the final immunization were assayed for DTx-specific IgG by ELISA as described in Materials and Methods. The geometric mean (bar) and individual values (open circles) are shown for each group.
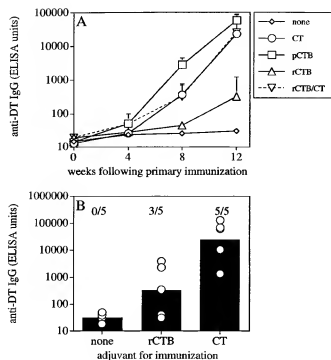
FIG. 4. Anti-DTx antibody response using CT, rCTB, or purified CTB as adjuvants. C57BL/6 mice ($n = 5$) were immunized on the skin with (50 µg of each) either CT, rCTB containing no CTA activity, pCTB with <1% residual CTA activity, or rCTB with 1% CTB added and DTx (100 µg) at 0, 4, and 8 weeks. Sera collected 4 weeks after each immunization were assayed for DTx-specific IgG by ELISA as described in Materials and Methods. In panel A, the geometric mean and the SEM are shown for each group, and the immune response is shown over time. In panel B, the comparative individual anti-DTx responses (open circles) and geometric mean (bars) using rCTB or CT alone are shown at 2 weeks after the third immunization. Responders per group (greater than twice the prebleed samples) are shown numerically.

or combinations enhanced the DTx-specific IgG titers, suggesting that the CTB subunit alone contains adjuvant function when applied to the skin (Fig. 4). However, when the adjuvant activity of either pCTB or the "spiked" rCTB was compared to that of the CT alone, the DTx titers elicited were significantly higher after the third immunization ($P < 0.05$). Thus, while the CTA subunit is not strictly required for adjuvant function, its presence is associated with optimal activity.

**Oligostimulatory DNA, "CpG" sequence, as an adjuvant for topically applied antigen.** Oligostimulatory DNA sequences have recently been shown to have adjuvant properties in intranasal and parenteral immunization approaches (5, 8, 23). To determine whether these short DNA molecules might also function as topical adjuvants, the recently published sequence 1826 (TCCATGACGTTCCTGACGTT, referred to as CpG or CpG) (33) was applied to the skin with DTx at 0, 4, and 8 weeks, and the resulting anti-DTx IgG titers were determined. Mice receiving topical applications of the CpG sequence with DTx developed elevated anti-DTx IgG responses in the serum, with kinetics that were similar to those observed in a separate cohort immunized with CT and DTx (Fig. 5A). One appealing feature of CT as an adjuvant is its ability to promote antibody responses to itself and coadministered antigens in mucosal tissues. Previous studies in this laboratory revealed that transcutaneous application of CT and DTx results in DTx-specific IgG in lung secretions and stool homogenates. As shown in Fig. 5C, animals immunized on the skin with the 1826 CpG

sequence and DTx also developed antigen-specific IgG in the lung secretions, as did the mice immunized with CT (Fig. 5B). Anti-DTx IgG was not detectable (≤0.1 at a 1:5 dilution) in lung wash samples from naive mice or animals immunized with an irrelevant control protein.

CpG sequences are associated with preferential induction of Th1 responses when introduced to the host by parenteral and intranasal routes (5, 8, 23). Based on these results, we hypothesized that topically applied CpG sequences might also enhance the Th1-associated humoral response, i.e., IgG2a, in the serum. However, application of CpG with DTx on the skin resulted in both increased anti-DTx IgG2a (Th1) and IgG1 (Th2) responses and, as observed with LT and CT, the IgG1 response was predominant (Table 3).

**LPS, cytokines, and other non-ADP-ribosylating compounds as adjuvants for antigen administered transcutaneously.** The ability of rCTB, which lacks ADP-ribosylating activity, to function as a topical adjuvant suggested that other non-ADP-ribosylating compounds might also be active on the skin. To test this concept, several compounds associated with adjuvant activity following parenteral, oral, or intranasal application were tested on the skin using DTx as the coadministered antigen. For screening adjuvants on the skin, a single dose was selected based on cost, availability, and a dose of CT (100 µg), the current topical standard. In the first experiment, IL-1β fragment (200 µg), IL-2 (1 µg), IL-12 (1 µg), and TNF-α (0.83 µg) were tested using CT (100 µg) and CpG sequence 1826 (100 µg) as positive controls and a non-CpG DNA sequence as a negative control. Anti-DTx IgG titers were ≤20 EU in all of the prebled samples and in the five mice receiving DTx alone or with the control DNA sequence. In the experimental groups, all of the adjuvants tested displayed some degree of activity on the skin as defined by the induction of anti-DTx titers of >4-fold over that observed in the highest average prebleed in that experiment (i.e., >40 EU for experiment 1 and >108 EU in experiment 2). Thus, following the third immunization, IL-1β fragment induced responses in two of five mice, IL-2 in three of five mice, IL-12 in three of five mice, and TNF-α in three of five mice. Screening of LPS (100 µg), CFA (diluted 1:4 with antigen solution), alum (100 µg), and MDP (100 µg) in a second experiment also revealed adjuvant activity for these compounds, with V. cholerae LPS yielding responses in four of five mice, Shigella LPS in three of five mice, CFA in four of five mice, alum in four of five mice, and MDP in two of five mice.

## DISCUSSION

TCI is based on the premise that systemic immune responses can be initiated by immune stimulation at the surface of the skin. The role of the adjuvant appears to be crucial for the induction of robust immune responses by this route (15, 17, 29). Although CT was used as the adjuvant in the earliest studies, we hypothesized that the immune stimulation could be provided by a variety of adjuvants known to use different mechanisms for activating antigen-presenting cells. In the present study, we confirm that TCI is not limited to CT or bARES with ADP-ribosylating exotoxin activity and does not require binding of ganglioside GM$_1$ by the B subunit of CT. Rather, a wide variety of adjuvants can act to stimulate the systemic immune responses that characterize TCI.

Previous studies had shown that LT could induce anti-LT responses when applied topically (17). LT and CT are homologous and share similar molecular organizations and binding activities. LT has been shown to act as an oral adjuvant in humans (27) and as an oral and nasal adjuvant in mice (21). In
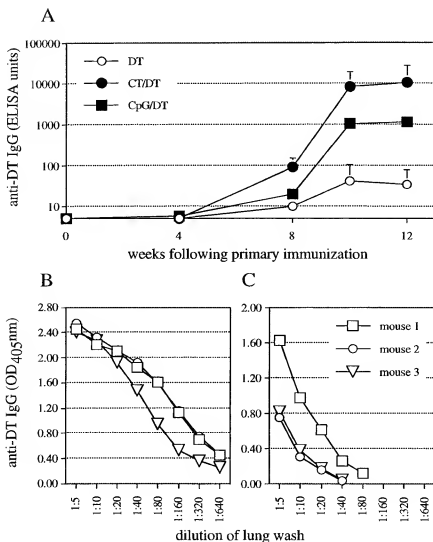
FIG. 5. Anti-DTx antibody response using CT or CpGs as adjuvants. C57BL/6 mice ($n = 5$) were immunized on the skin with either CT or CpGs (100 µg each) as adjuvants and DTx (100 µg) at 0, 4, and 8 weeks. Sera collected 4 weeks after each immunization were assayed for DTx-specific IgG by ELISA as described in Materials and Methods. In panel A, the geometric mean and the SEM are shown for each group, and the immune response is shown over time. In panel B, the individual titration curves for mice immunized with CT+DTx are shown for the lung washes from three mice sacrificed at 4 weeks after the third immunization. In panel C, the individual titration curves for mice immunized with CPGs+DTx are shown for the lung washes from three mice sacrificed at 4 weeks after the third immunization.

the present study, LT acted as an adjuvant for topical immunization using DTx as a test antigen (Fig. 1). The use of LT as an alternative to CT and the essential role of the adjuvant for the induction of robust immune response were more extensively demonstrated using TTx as the antigen (Fig. 2). In fact, the addition of as little as 1 µg of LT stimulated a several-log increase in anti-TTx antibody titers, thus illustrating the important role that the adjuvant plays in the immune response induced using TCI. Additionally, studies using CT had shown that antibodies induced to coadministered antigens as well as CT were functional in protection models (16, 17). Anti-TTx antibodies induced by LT were similarly protective in the tetanus challenge model, with solid protection resulting from as little as 5 µg of LT and 5 µg of TTx (Table 2).

To extend these findings, mutants of LT devoid of ADP-ribosyltransferase activity or devoid of this activity or with inactive trypsin cleavage sites were evaluated as adjuvants for the test antigen DTx. These adjuvants are thought to have

TABLE 3. Serum anti-DTx subclass analysis in mice immunized with CT and DTx or with CpG and DTx[a]

| Immunization group | Mean IgG concn (µg/ml) | | IgG1/IgG2a ratio |
|---|---|---|---|
| | IgG1 | IgG2a | |
| Expt 1 | | | |
| CT+DTx | 172.7 (65.8–516.2) | 10.2 (4.3–18.0) | 17 (4–35) |
| CpG1+DTx | 6.8 (0.7–109.7) | 1.1 (0.3–6.9) | 13 (12–16) |
| Expt 2 | | | |
| CT+DTx | 136.7 (19.2–612.2) | 10.1 (2.4–37.7) | 13 (8–21) |
| CpG1+DTx | 4.2 (2.6–8.5) | 0.2 (0.0–0.8) | 21 (11–94) |

[a] C57BL/6 mice ($n = 5$) were immunized on the skin with CT and DTx (100 µg each) or with CpG1 and DTx (100 µg each) at 0, 4, and 8 weeks. Serum collected 4 weeks after the final immunization was assayed for DTx-specific IgG1 and IgG2a by ELISA as described in Materials and Methods. Sera from unimmunized mice or animals immunized with adjuvant or antigen alone contained undetectable levels of DTx-specific antibody (≤1 ng/ml [IgG1], 0.5 ng/ml [IgG2a]). Values in parentheses are ranges.

TABLE 4. Use of non-ADP-ribosylating exotoxins as adjuvants for DTx administered by TCI[a]

| Expt and adjuvant (µg) | Anti-DTx IgG (EU) | | | | | | No. of positive mice/total no. |
|---|---|---|---|---|---|---|---|
| | Pre | 1 | 2 | 3 | 4 | 5 | |
| Expt 1 | | | | | | | |
| Control DNA sequence (100) | ≤10 | 19 | 12 | 5 | 5 | 11 | 0/5 |
| CT (100) | ≤10 | 22,026 | 47,526 | 13,285 | 113,894 | 140,058 | 5/5 |
| Immunostimulatory CpG (100) | ≤6 | 1,171 | 22,750 | 4,124 | 126 | 115 | 5/5 |
| IL-1β fragment (200) | ≤5 | 670 | 149 | 8 | 10 | 10 | 2/5 |
| IL-2 (1) | ≤2 | 13 | 111 | 345 | 49 | 35 | 3/5 |
| IL-12 (1) | ≤4 | 57 | 425 | 43 | 5 | 17 | 3/5 |
| TNF-α (0.83) | ≤1 | 1,808 | 830 | 7 | 1,477 | 7 | 3/5 |
| Expt 2 | | | | | | | |
| None | ≤13 | 31 | 16 | 9 | 6 | 23 | 0/5 |
| CT (50) | ≤10 | 4,887 | 76,282 | 201,637 | 1,027 | 2,848 | 5/5 |
| LT (50) | ≤27 | 35,603 | 16,886 | 37,472 | 5,621 | 5,007 | 5/5 |
| Shigella LPS (100) | ≤10 | 311 | 198 | 16 | 261 | 10 | 3/5 |
| V. cholerae LPS (100) | ≤12 | 122 | 768 | 111 | 1,066 | 50 | 4/5 |
| CFA (1:4 with antigen solution) | ≤16 | 630 | 1,905 | 106 | 294 | 996 | 4/5 |
| Alum (100) | ≤5 | 648 | 81 | 340 | 801 | 109 | 4/5 |
| MDP (100) | ≤10 | 512 | 44 | 548 | 23 | NA | 2/4 |

[a] Mice (6 to 8 weeks of age) were immunized on the skin with DTx (100 µg) and the indicated adjuvants at 0, 4, and 8 weeks. At 12 weeks after the primary immunization, the animals were bled, and the anti-DTx titers were determined using an ELISA. The results are reported in EU, which are defined as the inverse of the dilution yielding an $OD_{405}$ of 1.0. Positively responding mice are defined by the induction of anti-DTx IgG titers >4-fold over that observed in the mean prebleed samples (i.e., >40 EU for experiment 1 and >108 EU for experiment 2). Pre, prebleeding titer for group. Numbers used as column heads refer to individual mice ($n$ = 5) in each group.

a lower propensity for adverse side effects. Although the relative potency of these adjuvants was not fully examined, it did appear that the absence of ADP-ribosyltransferase activity decreased the magnitude of the immune response to DTx (Fig. 3). Similarly, rCTB, which is also devoid of ADP-ribosyltransferase activity, was used as the adjuvant, anti-DTx antibody responses were of lower magnitude than those of the CT holotoxin-adjuvanted group. When a small amount of holotoxin was added to the rCTB, the adjuvant activity was restored and the spiked rCTB had a potency comparable to that of CT alone (Fig. 4). This concept is further reinforced by the observation that purified CTB, which contains a small amount of holotoxin, was also equipotent as an adjuvant with the holotoxin. In another study, the use of CTA alone as an immunogen suggested that the B subunit was important for potent adjuvanticity (17). The relative contribution and mechanisms underlying the adjuvant activity of CT, LT, their mutants, and subunits are debated in the context of nasal and oral delivery (2, 13, 26), but the data presented here support their use as adjuvants for TCI and thus provide several options that can circumvent the potential problems with holotoxins.

To further probe the general observation that TCI is not restricted to CT, we evaluated a wide range of readily available adjuvants and cytokines for adjuvant activity in a topical application. LPS, TNF-α, IL-1β, and CpGs (22) are known to be activators of Langerhans cells, and IL-12 is a product of activated Langerhans cells. IL-2, alum, CFA, and MDP are well-known adjuvants with different mechanisms of immune stimulation (31). As shown in Table 4, each adjuvant stimulated a response to the coadministered antigen that was greater than that with antigen alone. It is important to note that this type of screening experiment does not optimize the formulation for delivery. The empiric dose, the antigen adjuvant ratio, and the aqueous vehicle may all have important effects on the efficiency of delivery and, therefore, the magnitude of the immune response elicited. The improvement of efficiency in delivery by optimization is illustrated by the results in Fig. 2, where as little as 1 µg of LT and 5 µg of TTx induced a robust anti-TTx

response in contrast to earlier studies (15), where the immunization was not optimized. Thus, each adjuvant considered for use in TCI would be expected to require optimization for efficient delivery, consistent responses, and potent immune stimulation.

There have been reports that LT and CT elicit qualitatively different immune responses; LT stimulates a Th1-type response, and CT reportedly stimulates a Th2 response (32). However, when the anti-DT antibody responses were evaluated for IgG subclass differences that might reflect a Th1-Th2 polarity, no difference between CT and LT was seen in the present study, and both IgG1 and IgG2a were produced, indicating a mixed immune response. Surprisingly, even CpGs, known for their strong propensity for Th1 polarity in T-cell assays (23) and in vivo models such as leishmaniasis (33), did not demonstrate a clear shift to greater amounts of IgG2a compared to the response elicited by CT or LT (Table 3). Clearly, the polar T-helper responses elicited may be dependent on many factors, including the route of delivery, the magnitude of immune stimulation, and the coadministered antigen. However, in the setting of TCI, mixed IgG subclass responses are elicited by CT, LT, and CpGs. CT is also known to stimulate cellular immunity to coadministered antigens in the form of cytotoxic T lymphocytes (28) and antigen-specific $CD4^+$ T-cell responses (18, 26), but the T-cell response to CT itself has yet to be characterized.

The use of topically applied vaccines may address the need for needleless vaccine delivery (19, 34) and decrease the barriers to immunization. In this study, we strengthen the observation that adjuvants play an essential role in the induction of a robust immune response to TCI. We further show that widely different adjuvants, in terms of composition and mechanisms of action, can be applied to the skin to induce responses to coadministered antigens. Although these studies have used DTx as a test antigen, we have found that a wide variety of antigens, including particles such as live viruses, can be delivered topically (20). The data presented here suggest that TCI embodies a broad observation that an antigen and adjuvant

applied to hydrated skin can induce potent systemic immune responses that may be expected to provide protection against vaccine-preventable disease. The several adjuvants described in this study, can be readily produced and inexpensive and, in several cases, can be safely circulated in the general population without side effects. Although there are significant challenges to the development of topical delivery of vaccines, TCI can be considered to be an important part of the array of immunization and vaccine delivery strategies.

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